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Morphometric, molecular, ontogenetic and demographic observations on selected populations of the Lizard Orchid, *Himantoglossum hircinum*

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[Running Head: *Bateman et al.* Observations on Lizard Orchid populations]

Himantoglossum hircinum is one of the rarer and more charismatic orchids in the British flora. Morphometric comparison of the two largest and best-known populations in southern England – the coastal dune population at Sandwich and the chalk grassland population at Newmarket – using 46 characters showed that they differ only subtly, the Sandwich plants being on average more vegetatively robust and slightly more darkly pigmented, but possessing less extensive lip-spots and substantially longer 'arms'. A comparatively morphologically divergent semi-desert population from Ifrane, Morocco differs from the English populations in having broader stems, less recurved 'arms', a more strongly down-curved spur and in lacking near-circular spots within the sepals. Molecular comparison of 46 plants, representing 13 English populations and 18 populations from Continental Europe and Morocco, revealed only subtle distinctions in the high-copy nuclear gene ITS, and smaller-scale comparisons of the low-copy nuclear (*LEAFY*) and plastid (four intron) regions proved to be even less discriminatory. These results reinforce prior morphological inferences that *H. hircinum* is a cohesive species. Scanning electron microscopy elucidated the ontogeny of these remarkable flowers, suggesting that the exceptionally elongate central labellar lobe originated by accelerated heterochronic growth and showing that the characteristic spiral torsion always runs counter-clockwise. Lateral fusion of the paired viscidia is convergent with several other lineages of subtribe Orchidinae. Review of pollination and life-history features of *H. hircinum* suggest that they are typical of food-deceptive species within Orchidinae. The Lizard Orchid is infamous for geographic mobility; its cycles of expansion and contraction through the last century have been interpreted as reflecting a net northward migration in response to recent climate change. Our data tentatively suggest relatively recent colonisation of Morocco at high altitudes and an overall northwestward direction of migration into the UK. ITS ribotypes indicate multiple immigration events leading to levels of genetic diversity in England comparable with those on the Continent. A non-recent origin is inferred for *H. hircinum* which, despite recent systematic revisions, may

harbour further cryptic species; the taxonomic status of supposed outlying populations in southern Italy in particular is questioned by the present genetic data.

Keywords: climate change; diagnostic characters; floral ontogeny; geographic distribution; *Himantoglossum hircinum*; Internal Transcribed Spacer; *LEAFY*; migration, Morocco, morphometrics; multivariate ordination; plastid, UK.

Introduction

Despite being widespread and locally common across much of western Europe, the robust and visually striking Lizard Orchid (*Himantoglossum hircinum* (L.) Spreng.: Fig. 2) is rare in the UK and was among the first tranche of 21 vascular plant species to be placed on Schedule 8 of the 1981 Wildlife and Countryside Act. It still appears in the *British Red Data Book*, where its national conservation status was downgraded from Vulnerable to Near-Threatened between 1999 and 2005 (cf. Farrell, 1999; Cheffings & Farrell, 2005). Brief scrutiny of the *British Plant Atlas* (Preston *et al*., 2002) revealed 19 post-1987 hectad records (excluding Jersey), scattered across a triangle of south-eastern England that extends westward to Berrow, Somerset and northward to Lakenheath, Suffolk; however, localities are concentrated particularly strongly in Kent (Fig. 1).

In England, the habitat preferences of the Lizard Orchid mirror those of the Pyramidal Orchid (*Anacamptis pyramidalis*): it prefers a shallow calcareous soil – either limestone or blown coastal sand rich in comminuted invertebrate shells – but is more tolerant than some grassland orchids of competition from longer grasses and scrubby bushes, and of periodic drought (Fig. 3). A further tolerance for soils disturbed within antiquity means than a significant proportion of its localities lie outside mainstream nature reserves; sites include roadside verges and even lawns (Fig. 3a, c). The predilection of *H. hircinum* golf courses and horse-racing courses in England is legendary, but in truth, such sites encompass only a minority of recent localities (Carey, 1999; Carey & Farrell, 2002).

The Lizard Orchid shows broadly similar habitat preferences in mainland Europe, where its core distribution almost precisely coincides with present-day France (Pfeifer *et al*., 2009; *contra* Carey & Farrell, 2002). Tongues protrude northwards into southern England and the Low Countries and northeastward into SW Germany. To the south, outliers occur to the southwest in northern Spain, southern Iberia and northern Morocco. In northern Italy, *H. hircinum* is considered to be replaced by its sister-species, *H. adriaticum*. However, *H. hircinum* is widely considered to reappear in southern Italy and Sicily, and reputedly also occurs in the Tunisian hills opposite Sicily.

There have been a few studies of pollination in *H. hircinum* but these include one that used this species as a model system for the study of geitonogamy – transfer of pollinaria between genetically identical flowers occupying the same inflorescence (Kropf & Renner, 2008). A few populations have also been subjected to unusually detailed studies of annual demographics (Carey, 1998, 1999; Heinrich, 2003; Pfeifer, 2004; Pfeifer *et al*., 2006a).

Perhaps the most impactful scientific investigations of *H. hircinum* have focused on its broader geographic distribution, fuelled by the pioneering arguments of Good (1936) that this species is particularly sensitive to, and thus is a credible model indicator of, climate change (e.g. Hartley *et al*., 2004). This assertion has been reinforced by recent autecological/demographic studies by Carey and colleagues (Carey & Brown, 1994; Carey, 1998, 1999; Farrell & Carey, 1999; Carey & Farrell, 2002; Carey *et al*., 2002) in the UK and Pfeifer and colleagues (Pfeifer, 2004; Pfeifer & Jetschke, 2006; Pfeifer *et al*.,

2006a, b, 2009, 2010) in SW Germany, where *H. hircinum* was designated the 'Orchid of the Year' in 1999 (Heinrich & Voelckel, 1999). Most of these studies have paid particular attention to the more northerly populations of *H. hircinum*, whereas no previous author has considered the southern-most occurrences of this species that occur in northwest Africa and were sampled for the present study.

Although species circumscription in the genus *Himantoglossum* has occasionally been addressed (cf. Delforge, 1999; Sramkó & Molnár, 2012; Sramkó *et al*., 2013), the process has been less rigorous than in several other genera of European Orchideae (cf. Bateman, 2013). In addition, the evolutionary origin and ontogenetic underpinning of the remarkable floral morphology of *H. hircinum* have received little attention relative to those of other genera (cf. Kurzweil, 1987; Box *et al*., 2008; Rudall *et al*., 2013).

Here, we offer a more rounded account of *H. hircinum*, utilising morphometric, molecular and microscopic techniques in order to better understand its species circumscription, infraspecific variation and floral evolution. We set these new observations in the context of previous interpretations of the biology and ecology of this charismatic orchid.

Materials and Methods

Morphometrics

Field sampling—The modest sampling for morphometric analysis targeted the two largest, longest-established, best-known and most carefully monitored English populations of *H. hircinum*, which also provided a useful contrast of the species' two preferred habitats: stabilised sand dunes at Sandwich, Kent and chalk grassland at Newmarket, Cambridgeshire (Figs 2, 3). Ten plants were measured in each of the two populations in June 2010. The site selected within the Sandwich population was located in short exposed grassland on a shallow west-facing slope along the line of dunes adjacent to the beach at *ca* 1 m asl (Fig. 3d). The smaller Newmarket population was concentrated in longer, quinquennially burned grassland clothing the steep south-facing bank of an ancient earthwork at *ca* 30 m asl (Fig. 3e). The even smaller comparative population from Morocco, examined in May 2012, was located 3 km southwest of Ifrane along the northern slopes of the Middle Atlas; it contrasted strongly in altitude with the other study sites, occurring at 1650 m asl. The semi-arid site was a subdued rocky hillock surrounded by sparse, goat-grazed dwarf scrub near the margin of extensive cedar forests (Fig. 3b).

Data collection—Our within-site sampling strategy was designed to minimise disturbance to individual plants. Within each population, plants for study were chosen to proportionately reflect the range of variation evident in both morphology and habitat. Vegetative characters were measured non-destructively from *in situ* plants, and only two flowers from each plant were removed for further study: the first was permanently mounted and measured (Fig. 4), whereas the second was placed in fine-grained dried silica gel to act as a DNA-friendly voucher. Wherever possible, the floret chosen to provide morphometric data on the flower, ovary and bract was located 30–40% of the distance from the base to the apex of the inflorescence, in order to minimise the effect of any diminution in flower size toward the apex.

The 46 characters scored (Table 1) described the stem and inflorescence (9), leaves (4), labellum (17), spur (3), lateral petals (2), lateral sepals (9) and gynostemium (2). They can alternatively be categorised as metric (31), meristic (5), multistate-scalar

(5) and bistate (5). The colours of the 'limbs' of the labellum, and of the reverse of the sepals, were matched to the closest colour block(s) of the Royal Horticultural Society Colour Chart (Anonymous, 1966) and later converted to three quantified variables recognised by the Commission Internationale de I'Eclairage. A more detailed account of the chosen characters and methods of measurement applied to the genus *Himantoglossum s.l.* will be given elsewhere (Sramkó *et al*., in prep.).

Data analysis—Data for individual plants were summarised in an Excel v14.2 spreadsheet. Means, sample standard deviations and coefficients of variation were calculated for every character in each of the three populations. Univariate analyses were summarised and presented using Deltagraph v5.6 (SPSS/Red Rock software, 2005).

The morphometric matrix contained 23 individuals × 46 characters. After combining the numbers of basal and bracteoidal leaves, and omitting four characters that varied among other species of *Himantoglossum* but were invariant within *H. hircinum*, the assembled data were analysed by multivariate methods using Genstat v11 (Payne *et al.*, 2008). All calculated ratios were also omitted from the multivariate analyses as, by definition, they duplicated their constituent characters.

The remaining 41 characters were used to compute a symmetrical matrix that quantified the similarities of pairs of data sets (i.e. plants) using the Gower Similarity Coefficient (Gower, 1971) on unweighted data sets scaled to unit variance. The matrix was in turn used to construct a minimum spanning tree (Gower & Ross, 1969) and subsequently to calculate principal coordinates (Gower, 1966, 1985) – compound vectors that incorporate positively or negatively correlated characters that are most variable and therefore potentially diagnostic. Principal coordinates are especially effective for simultaneously analysing heterogeneous suites of morphological characters and can comfortably accommodate missing values; they have proven invaluable for assessing relationships among orchid species and populations throughout the last three decades (reviewed by Bateman, 2001).

Molecular analyses

Experimental details of molecular data acquisition were given by Sramkó *et al*. (2013). Here, we focus on the preceding sampling strategy and succeeding data analysis.

In addition to the two populations studied morphometrically, a further 11 English localities were sampled for molecular analysis, encompassing the entire present geographic range of the species in the British Isles (Fig. 1); at these additional sites, observed population size ranged from *ca* 30 flowering plants to single transient individuals. A further 16 localities were sampled from Continental Europe in Spain (2 populations), southern France (5), northern France (3), southwest Germany (4), southern Italy (1) and Sicily (1); also, two localities were sampled in the Middle Atlas Mountains of Morocco (Fig. 6). Together, these samples spanned the full geographical range of *H. hircinum*.

DNA was extracted from a portion of each desiccated flower following the 2× CTAB (cetyltrimethyl ammonium bromide) procedure (Doyle & Doyle, 1990). The main molecular survey was conducted by direct-sequencing the Internal Transcribed Spacer (ITS) of the nuclear ribosomal DNA, seeking inter- and infra-populsation variability that could be of phylogeographic as well as taxonomic vale (e.g. Kay *et al*., 2006). Subsampling allowed preparation of between one and four individuals from each of 13 localities in the UK and 17 non-UK sites. Direct sequencing of ITS (performed in Korea by Macrogen) in a total of 46 plants revealed frequent individuals that showed additive polymorphic sites in ITS. Hence, one sample from three selected populations of *H.*

hircinum (Winterbourne, near Bristol in the UK, Fayence in the Var region of southeast France, Capizzi in north-central Sicily) was subsequently cloned, yielding a minimum of six clones (Sramkó *et al*., 2013). Most variable sites were single nucleotide polymorphisms, but ITS 'populations' within five plants were length polymorphic (i.e. reflected indels) that could be categorised but could not be assigned to specific ribotypes using Collapse v1.2 or inserted into phyletic networks generated using TCS v1.21 (Clement *et al*., 2000).

These ITS data were then combined with ITS sequences obtained from all other taxa within *Himantoglossum s.l.*, plus *Steveniella satyrioides* as outgroup, to provide a broad phylogenetic context for the present study. Phylogenetic tree reconstruction based on maximum parsimony was performed using PAUP v4.0b10 (Swofford, 2003), employing default settings for heuristic search and subsequent bootstrap analysis with 1000 pseudo-replicates.

Further molecular investigations, conducted as part of a broader phylogenetic study of the genus *Himantoglossum sensu lato* (Sramkó *et al*., 2013), included five samples of *H. hircinum* that were selected to span the full geographic distribution of the species: Newmarket (England) to the northwest, Habkirchen (SW Germany) to the northeast, Grasse (SE France) representing the core distribution, Pacios (NW Spain) representing the northern Iberian outlier, and Calvello (S Italy) representing the southern Italian outlier. These samples were sequenced for the first intron of the low-copy (and developmentally crucial) nuclear gene *LEAFY* and for three rapidly mutating and phylogenetically informative plastid regions: the *accD–psaI* intron, the *psbA–trnH* intron, and two introns of the *trnL–ndhF* region, specifically *trnL–rpl32* and *rpl32–ndhF*.

For the genus-wide analysis, raw sequences were edited and aligned by eye using BioEdit v7.1.3 (Hall, 1999). Following ILD testing via a 100-replicate partition homogeneity test in PAUP, the four plastid regions were combined into a single aggregate matrix. Phylogenetic tree reconstruction based on maximum parsimony was again performed using PAUP v4.0b10 (Swofford, 2003), employing default settings for heuristic search and subsequent bootstrap analysis with 1000 pseudo-replicates. All analyses were run on Bioportal (Kumar *et al*., 2009). However, when the taxonomically broad master matrices were reduced to *H. hircinum* only, algorithmic analysis (whether tree building or ordination) of the resulting datasets was unnecessary, as the DNA sequences were sufficiently similar to be compared by eye.

Scanning electron microscopy

An entire inflorescence of *H. hircinum*, presenting both opening flowers and unopened buds, was sampled from the seed-derived stock of a private orchid grower in Gloucestershire, England. Material was preserved in 70% ethanol before being processed for scanning electron microscopy (SEM). Buds were removed from the inflorescence and dehydrated through an ethanol–water series to 100% ethanol. Samples were dried in a Tousimis Autosamdri 815B critical-point dryer (CPD) using carbon dioxide as the carrier gas. Flowers were mounted onto stubs using double-sided adhesive discs and dissected under a Wild Heerbrugg M7A microscope. Partially dissected samples were coated in platinum using an Emitech K550 sputter coater and imaged using a Hitachi S-4700 II cold-field emission scanning electron microscope (SEM). For each bud, multiple images were captured and, where appropriate, later aggregated into colourised composite reconstructions using Adobe Photoshop.

Results

ITS ribotypes

The ITS phylogeny (Fig. 5) confirmed the topology for *Himantoglossum s.l.* (i.e. including the former genera *Comperia* and *Barlia*) derived by Bateman et al. (2003), and placed the Caucasian endemic *H. formosum* in the predicted position as sister to the remaining taxa of *Himantoglossum s.s.* (Sramkó et al., 2013). However, no reliable phylogenetic structure was identified within this residual clade, which identified only single autapomorphic states in ITS supporting the putative eastern Mediterranean endemics *H. montis-tauri* and *H. galilaeum* (Fig. 5).

Nonetheless, two ribotypes were shown by Sramkó *et al*. (2013) to dominate the *H. hircinum-adriaticum* and *H. jankae-caprinum* groups, respectively: RGa1 to the west of the Bosphorus and RGb1 to the east (note that, prior to 2012, *H. jankae* and *H. caprinum* were widely known as *H. caprinum* and *H. affine*, respectively: Molnár *et al*., 2012a; Sramkó *et al*., 2012). Of 51 positions variable within the *H. hircinum-caprinum* aggregate, 20 varied within *H. hircinum*; indeed, *H. hircinum* yielded more ribotype variants that its sister species, *H. adriaticum*, which was dominated by the core ribotype RGa1 (Fig. 6). Most of these variants, including RGa8 found at Masa in the mountains of northern Spain, deviated from the core ribotype RGa1 by just one single-nucleotide polymorphism or indel. In addition, the La Chapelle population in the Vercors region of southeast France contained a length-polymorphic variant (L05), and that from the nearby Grasse locality in the Var yielded both a further length-polymorphic variant (L02) and a putative pseudogene.

Similar levels of variation similar to those on the Continent are evident among the UK populations of *H. hircinum* (Fig. 6). At least 11 of the 13 sequenced populations contained the dominant western European ribotype RGa1, but those from Winterbourne and Newmarket also contained a single-base-pair deviant RGa6 and the isolated plant from the Kent coast at St Margaret's yielded another ribotype group, RGa5, that also appears to be unique to England. In addition, the length-polymorphic L05 ribotype previously observed in the Vercors was also found in Royal St George's golf course at Sandwich, while sporadic occurrences of ribotype group RGa3 stretched from Sicily to Hythe via Calais (Fig. 6a–c).

The most substantial ITS divergence was found in southern Italy and especially Sicily, where the supposed outlying populations of *H. hircinum* yielded two more strongly divergent ribotypes that are atypical not only of the core distribution of *H. hircinum* but also of *H. adriaticum*, its sister species that characterises central and northern Italy and parts of the Balkans (Sramkó et al., 2013). Specifically, the Capizzi population from north-central Sicily was dominated by a more divergent ribotype RGa4 that differed in three nucleotides, also incorporating the length-polymorphic group L04 and group RGa3, which also characterised the other southern Italian population sampled, Calvello.

Surveying the data at the lower hierarchical level of specific ribotypes showed some intriguing similarities within the frequent RGa1 category. Ribotype H30 is shared by plants from Slepe (Dorset, UK), Newmarket (Cambridgeshire, UK) and Camber (East Sussex, UK), and ribotype H29 by plants from Berrow (Somerset, UK), Broadstairs (NE Kent, UK) and Fayence (the Var region of SE France). Most intriguingly, ribotype H54 occurs in Guildford (Surrey, UK), Isleworth (London, UK), Sandwich (E Kent, UK) and Reviers (Normandy, France). However, these ribotypes are so closely similar that it would be an error to over-interpret their patterns.

Plastid haplotypes

The tree generated from the four-intron plastid matrix confidently placed the six analysed accessions of *H. hircinum* as sister to three accessions of *H. adriaticum*. Four of the *hircinum* accessions (UK, Spain, France, Germany) yielded identical sequences. Those from southern Italy (Calvello) and Morocco (Ifrane W) deviated by a single base-pair in *trnL-ndhF*, though the Moroccan accession also exhibited three autapomorphic indels in the *accD-psaI* intron (for a more detailed account of the plastid and following *LEAFY* regions see Sramkó *et al*., 2013).

Low-copy nuclear gene (*LEAFY*)

LEAFY sequences also reliably distinguished *H. hircinum* from *H. adriaticum* as a well-supported clade, but showed remarkably little within-species variation. The Spanish accession was tentatively placed as sister to the remaining samples on the basis of just one base-pair difference, and the accession from Grasse (SE France) apparently possessed two autapomorphic states. Unfortunately, we were unable to obtain *LEAFY* from the southern Italian populations as the DNA was of insufficient quality.

Morphometric comparison

The morphometric matrix of 23 plants × 46 characters contained 27 (2.5%) missing values. Of these absences, 14 represented leaf dimensions of the Newmarket populations, which were severely desiccated by the time of measurement (in contrast, leaves often persist into the flowering period at Sandwich: Carey & Farrell, 2002). A further ten missing values represented the length of lower bracts at the Sandwich population – a character that was introduced into the study only after this pioneering dataset had been acquired. Moreover, within the context of this particular analysis, four characters proved to be invariant: the reliable *ca* 45° angle of the labellar 'torso' relative to the stem, together with the lateral sepals being connivent into a hood and bearing brownish-purple lines as both marginal strips externally (abaxially) and dashes internally. In addition, only one study plant (from Newmarket) exhibited a 'tail' mid-way between the 'legs', and it was less than 1 mm long. Populations means, sample standard deviations and coefficients of variation are given in Table 1 for each of the study populations.

The resulting plot of the first two principal coordinates (Fig. 7) encompasses a respectable 56% of the total variance and readily distinguishes all individuals of each of the three study populations; the superimposed minimum spanning tree (MST) in particular identifies the two English populations as being more similar to each other than either is to the Moroccan population. The three populations appear roughly equally cohesive on the plot, the weaker MST links within the Ifrane population being attributable to the smaller number of plants available for statistical comparison.

When individual characters are considered, several serve to distinguish Ifrane from the English populations (Table 1). Vegetatively, the stem is more robust relative to its height (Figs 2c, 8) and there is a stronger disparity in length between the bracts of the lowermost flowers and bracts occurring higher up the inflorescence. Turning to labellum shape and size, Ifrane superficially appears to have longer 'legs', but its higher mean value actually reflects the presence of just one plant possessing an exceptionally deeply divided central lobe (Fig. 9). Also, the 'arms' of Ifrane labella tend to be less strongly recurved relative to the plane of the 'torso' (Fig. 2). The provision of a marginally longer spur appears to encourage greater downward curvature and the column is somewhat wider. Most strikingly, the Ifrane population lacks purple spots within the sepals (a feature also absent from one sampled plant at Newmarket), though the Ifrane plants share with the English populations the presence of purple dashes (Fig. 2). The Moroccan

population generally showed greater variation in floral dimensions but less variation in vegetative dimensions compared with the English populations (Table 1).

Morphological differences distinguishing the two English study populations are more subtle (Table 1). The Newmarket population has the narrowest stem relative to its height (Fig. 7) and is less floriferous. Its flowers have somewhat narrower lateral sepals but somewhat broader lateral petals. More strikingly, the 'arms' of Sandwich plants are on average longer than those of Ifrane and double the length of those from Newmarket (Figs 2, 9), a fact that is particularly evident from representative labellar silhouettes (Fig. 4) – indeed, the mean 'arm' length of Sandwich plants of *H. hircinum* matches that of *H. jankae* from eastern Europe (cf. Sramkó *et al*., 2012). The remaining differences reflect anthocyanin pigmentation. The upper portions of the stems of plants from Sandwich are more reliably stained purple than those from Newmarket (*ca* 40% versus *ca* 90%). Careful colour-matching showed that the labellar margin of Sandwich plants is a slightly deeper purplish-brown, and the outer surfaces of the sepals are a somewhat deeper and bluer green (Fig. 2). In contrast, the discrete purple papillate spots tend to be slightly fewer and more localised in the centres of Sandwich labella.

Floral ontogeny

All of the structures that will eventually constitute a mature *H. hircinum* flower *ca* 50 mm long are already evident in a bud a mere 2 mm long (Fig. 10a). Early growth favours the gynostemium, whereas later expansion of the remaining structures (ovary, auricles, labellar lobes, spur) occurs at approximately equal rates (cf. Fig. 10a–c). Although there is equivocal evidence that expansion of the labellar lobes continues beyond those of other structures, labellum elongation appears to be consistently in advance of that shown by the equivalent developmental stages earlier divergent species of *Himantoglossum s.l.* The famed tightly-packaged 'watch-spring' morphology of the central labellar lobe is clearly visible even in early stages of growth, and is later mirrored (albeit in less tight spirals) by coiling in the lateral lobes. Lastly, the crenulations that reliably adorn the 'shoulders' of the lateral lobes become evident only comparatively late in ontogeny (Fig. 10c), presumably resulting from differential expansion of the labellum margins. Measurements of individual cells in contrasting developmental stages suggest that the enlargement of contrasting floral structures primarily reflects cell division rather than cell expansion.

Comparison of immature (Fig. 11a) with mature (Fig. 11b) labella reveals that the perfectly aligned longitudinal ranks of pavement-style epidermal cells overlying the mid-vein in early developmental stages later form a seemingly chaotic melange of highly papillate cells, also demonstrating that the purple spots on the labellum (Fig. 2e–g) are localised concentrations of anthocyanins within a single extensive papillate region rather than isolated clusters of papillae. The early stages of circinnation of the labellum are well-illustrated in Figure 11a, but only the later-stage Figure 11b features the plicate 'shoulders' of the lateral lobes that characterise the mature flower.

Discussion

Detailed morphology of H. hircinum *in the UK*

Some authors have in recent years continued to implicitly or explicitly treat all members of the *H. hircinum-adriaticum* and *H. jankae-caprinum* aggregates as just one or, at most, two species (e.g. Sundermann, 1973, 1980; Moore, 1980; Carey & Farrell, 2002; Foley & Clarke, 2005). However, other authors recognised larger numbers of species (e.g. Nelson, 1968; Landwehr, 1977; Teschner, 1980; Delforge, 1999, 2006); current evidence strongly

supports the recognition of up to 12 species within the expanded genus *Himantoglossum s.l.* (Sramkó *et al*., 2013, in prep.).

Given such taxonomic interest, and the appreciable levels of morphological and genetic diversity demonstrated within *H. hircinum* (Figs 2, 5, 6), surprisingly few previous authors commented on morphological variation among British populations. The main exception was Ettlinger (1997), who correctly observed variations in labellum shape (especially the length of the 'arms' and the depth of the sinus separating the 'legs') and pigmentation, notably amount of purple spotting in the centre of the labellum and the depth of the brownish-purple staining around its periphery. In addition, an anthocyanin-poor individual from Newmarket illustrated by Harrap & Harrap (2009, p. 353) vaguely echoes the characteristically unmarked and/or pale-flowered species of *Himantoglossum s.s*. that occur around the eastern Mediterranean (cf. Sramkó *et al*., 2012, 2013).

When measured against our detailed morphometric data (albeit representing only three populations: Table 1), descriptions of *H. hircinum* in the literature have generally proven to be more accurate than those of most other species of Orchidinae (cf. Davies *et al*., 1983; Sell & Murrell, 1996; Carey & Farrell, 2002; Delforge, 2006; Harrap & Harrap, 2009). Nonetheless, inaccuracies are evident. An overly short range of 7–10 mm attributed to the sepals by Moore (1980) was reproduced in several subsequent descriptions (e.g. Sell & Murrell, 1996; Carey & Farrell, 2002; Foley & Clarke, 2005). A typographic error in Sell & Murrell (1996) permitted labella to be as little as 3 mm long! Some authors gave ranges of overall labellum length that fail to encompass the shorter end of the observed spectrum (e.g. Lang, 2004; Stace, 2010) whereas others neglect the longer end of the spectrum (e.g. Foley & Clarke, 2005). Similarly, some authors attempted to restrict spur length to less than 2.5 mm (Davies *et al*., 1983; Foley & Clarke, 2005) when it actually averages 2.7 mm and can reach 3.5 mm. Other authors under-estimated either the length of the ovary (Sell & Murrell, 1996; Carey & Farrell, 2002) or the width of the bracts (Carey & Farrell, 2002); yet others over-estimated the proportion of total plant height occupied by the inflorescence (Harrap & Harrap, 2009). Some authors offered as a diagnostic character the presence of a notch at the apex of the central lobe (Delforge, 2006; Stace, 2010) but ignored the *ca* 10% of plants that lack this notch. Lastly, Stace (2010, p. 880) noted that the leaves may be "purple-mottled", a statement that presumably was intended to instead refer to the upper portion of the stem; purple anthocyanins suffuse the stem within and immediately below the inflorescence in *ca* 40% of individuals, but we have never seen these vegetative pigments extend into the leaves of *H. hircinum*.

It is, of course, likely that our own limited morphometric measurements have failed to capture the full spectrum of morphology presented by *H. hircinum*, as well as having inevitably been modified by ontogenetic and ecophenotypic factors (cf. Bateman & Denholm, 1989). For example, plants measured by us previously at Sandwich in dune-slacks located further from the beach were taller than the 32 ± 7 cm (n = 10) reported here, averaging 47 cm in 1979 (n = 39) and 41 cm in 1981 (n = 27) – similar fluctuations in plant height between years were reported in a Hungarian population of *H. adriaticum* by Bódis & Molnár (2009). The largest plant of *H. hircinum* that we have encountered in the UK – an isolated individual that appeared briefly at Headley Warren, Surrey – measured 100 cm and bore 66 flowers in 1979 (Fig. 2b). Even larger plants, carrying up to 200 flowers, occur occasionally in both the UK and the Mediterranean region (Carey & Farrell, 2002; Pfeifer *et al*., 2009).

Contribution of* H. hircinum *to examples of phenotypic convergence

The pollinaria of *Himantoglossum s.s.* are distinguished by unusually robust columnar caudicles terminating in more-or-less hexagonal viscidia that are enclosed in a desiccation-resistant hemispherical bursicle and are fused laterally, such that both pollinia can only be transferred by pollinators as a single pollinarium (nonetheless, deposition on the stigma of pollinium fragments is sufficient to ensure adequate pollination: Carey & Farrell, 2002). Such lateral fusion of viscidia shows considerable parallelism within the phylogeny of Orchidinae (we are intrigued to know whether it is congenital or postgenital in *Himantoglossum*, but this would require access to even earlier stages of floral ontogeny). As well as characterising all species of *Himantoglossum s.l.* other than the basally divergent *H.* (formerly *Comperia*) *comperianum* (cf. Delforge, 1999; Claessens & Kleynen, 2011), fused viscidia are also evident in *Orchis* (formerly *Aceras*) *anthropophora*, *Anacamptis pyramidalis*, and all species of *Serapias*. Together, these taxa constitute a remarkable case of morphological parallelism.

In addition, the typical examples of flowers from our study populations illustrated in Figure 4 show that the labellum of *H. hircinum* is intermediate in outline shape between those of the mid-Mediterranean *H. adriaticum* and eastern European *H. caprinum* (formerly *H. affine*) (see Nelson, 1968; Delforge, 1999; silhouettes reproduced in Davies *et al*., 1983, p. 137; Delforge, 2006, p. 351). However, molecular data suggest that an origin of *H. hircinum* through hybridisation of *H. adriaticum* and *H. caprinum* is highly unlikely, thus indicating that labellum outline is also subject to morphological convergence within the genus.

A chromatographic (HPLC) analysis by Strack *et al.* (1989) of the floral anthocyanins of *H. adriaticum*, sister to *H. hircinum*, revealed a significant percentage of unknown compounds but a spectrum of identifiable pigments that was dominated by Serapianin and Seranin. These pigments also characterise *Himantoglossum* subgenus *Barlia*, *Serapias* and *Anacamptis papilionacea* (a species that is to some degree also convergent on *Serapias* in floral morphology). Moreover, the somewhat denser floral pigments of the Sandwich population of *H. hircinum* relative to the Newmarket population (cf. Fig. 2f, g) may simply reflect the epigenetic influence of the substrate in which they are rooted. Species of other genera of Orchideae, notably marsh-orchids of the genus *Dactylorhiza*, also routinely generate richer colours (presumably reflecting greater concentrations of anthocyanins) when growing in dune sands (cf. Bateman & Denholm, 1985).

Evolutionary-developmental aspects of floral ontogeny

The early stages of floral development in *H. hircinum* (Figs 10, 11) broadly follow those documented for other genera of Orchideae (Kurzweil, 1987; Box *et al*., 2008; Rudall *et al*., 2013). The greater size of the labellum of the *H. hircinum-jankae* clade does not simply represent giantism, as changes in its dimensions are non-allometric; the central lobe increases greatly in length, whereas its width decreases relative to those of sister-species *H. formosum* and *H. robertianum*. Thus, a shape change accompanies the size increase, showing that these changes can be attributed to the category of heterochrony known as peramorphy. We cannot entirely rule out some modest effects from precocious onset of labellum extension (pre-displacement *sensu* Alberch *et al*., 1979; see also Box *et al*., 2008) or delayed offset of growth (hypermorphosis), but the buds illustrated in Figure 9 suggest that the main contributor to the evolutionary elongation of the labellum is greatly increased growth rate (acceleration). We assume that growth rate is especially great on the ‘shoulders’ of the labellum, thereby generating the distinctive crenulations (Fig. 11b).

The final event in the ontogeny of the flowers is the uncoiling at anthesis of the elongate labellum and the immediate replacement of the planar coiling of the central lobe with a spiral twist (Fig. 2d). The direction of spiral (chirality *s.l.*) was coded as a morphometric character in the present study but ultimately failed to contribute to the multivariate analyses, as it rapidly became clear that all individuals of all species of *Himantoglossum s.s.* spiral counter-clockwise as viewed from the apex (Fig. 2). This consistent result presents a stark contrast to the helical inflorescences of orchids such as the Autumn Ladies-tresses (*Spiranthes spiralis*); large populations of this species maintain approximately equal numbers of plants with inflorescences that spiral in clockwise and counter-clockwise directions, reportedly encouraging allogamy via pollinating bees (Schilthuizen & Gravendeel, 2012; also B. Gravendeel, pers. comm., 2011; M. Mehrink, pers. comm., 2011). However, the helix of the *Himantoglossum* labellum does mirror the consistent direction of spiral in the lateral sepals of *Cypripedium* species reported by Welch (1998).

Pollination biology

In their comprehensive summary of pollinators of European orchid species, Claessens & Kleynen (2011) reported that 13 bee species (seven belonging to the genus *Andrena*: see also Teschner, 1980; Vöth, 1990; Kropf & Renner, 2008) and one species of *Oedemera* beetle have been observed removing pollinaria from plants of *H. hircinum*. Davies *et al.* (1983) and Carey & Farrell (2002) suggested that flies or hoverflies may also act as pollinators; certainly, taxonomically broader spectra of pollinators have been recorded in other species of *Himantoglossum* (Delforge, 2006; Claessens & Kleynen, 2011).

The pungent goat-like scent of the flowers, presumed to be a pollinator attractant, was initially ascribed to capronic acid (Kerner, 1891; Schmid, 1912), but a subsequent study combining gas chromatography with mass spectrometry revealed the scent to consist of two forms of decenoic acid plus lauric acid (Kaiser, 1993). The prominent, dark purple papillate spots adorning the 'body' of the labellum below the stigma have been said to operate as scent-secreting osmophores (Vogel, 1990). Certainly, our observations that the papillae are large, extensive and late-formed during ontogeny (Fig. 10) are all consistent with a likely function as osmophores.

A debate begun by Darwin (1877) regarding whether *H. hircinum* offers a nectar reward or alternatively operates entirely by food deceit is ongoing even today (Bateman, 2013). Evidently desperate to rebut the food-deceit theories of Fritz Müller, Charles Darwin speculated that a modest nectar reward could be obtained by insects by penetrating the tissues of the spur, an interpretation later discussed by Teschner (1980). In addition, Vöth (1990) argued that the dense papillae that partially obscure the spur entrance may secrete tiny drops of nectar, and several subsequent authors have also at least tentatively inferred the presence of a small nectar reward in *H. hircinum* (Neiland *et al*., 2001; Bournérias & Prat, 2005; Inda *et al*., 2012). In truth, such allegations could be levelled against any food-deceitful species of Orchidinae; there is little doubt that pollinators of *Himantoglossum* species receive no meaningful reward (Carey & Farrell, 2002; Kropf & Renner, 2008; Claessens & Kleynen, 2011; Bateman, 2013).

Similarly, there is little evidence of autogamy in *H. hircinum*, either through direct observation or through exploration of population-genetic structure. However, the occurrence of at least some geitonogamy (cross-pollination of genetically identical flowers occupying the same inflorescence) was confirmed long ago by the fact that even solitary flowering plants often set seed (Summerhayes, 1951). More recently, detailed observations by Kropf & Renner (2008) estimated a geitonogamy frequency of 36% in three populations of Lizard Orchid in southern Germany. As concluded by Kropf &

Renner (2006, p. 506), "foraging behaviour results in a mix of geitonogamy, near-neighbour pollination, and occasional long-distance outcrossing not fundamentally different from the situation in many other insect-pollinated perfect-flowered rewarding angiosperms." We suspect that geitonogamy frequencies of about one third are typical of most allogamous species of Orchideae.

Life history

Harrap & Harrap (2009) noted an average seed-set of *ca* 30% in UK populations, though figures differ considerably between years (Carey, 1999; Neiland *et al*., 2001; Carey & Farrell, 2002; Carey *et al*., 2002; see also data on *H. adriaticum* by Bódis & Molnár, 2009). Claessens & Kleynen (2011) reported that 18–62% of capsules set seed in 12 German populations, but fruit set was lower (6–7%) in the German population studied in detail by Pfeifer (2004). No seeds formed in a Dutch population examined by R. Wielinga, whereas seed-set reached 95% in one Italian population studied by Pfeifer *et al*. (2009). Individual capsules have been estimated to average just 1,200 seeds (Summerhayes, 1951; Carey, 1999). This is a small number relative to most other species of Orchidinae; given the comparatively large size of the capsules and the comparatively small size of the seed (testa = *ca* 340 × 120 μm), Carey & Farrell (2002) may have been right to suggest that typical seed numbers are higher.

Seed matures within 6–8 weeks of pollination (Farrell & Carey, 1999) and often germinates immediately, though periods of seed dormancy of up to three years have also been documented (Carey & Farrell, 2002). Germinated seeds typically generate a first leaf three years later and reach flowering size in a further three to five years (Rasmussen, 1995; Pfeifer, 2004; Pfeifer *et al*., 2006b; Harrap & Harrap, 2009). The resulting leaf rosettes are wintergreen (Carey, 1999; Carey & Farrell, 2002), allowing the plant to grow rapidly in the spring but often leading to senescence of the leaves by the time that the flowers open – a common feature of wintergreen species of Orchideae. Pfeifer (2004) reported a half-life of approximately five years (a figure typical of tuberous European orchids), the life expectancies of individuals being increased by periods of flowering and/or dormancy. Individual plants are known to have survived for at least 19 years (Harrap & Harrap, 2009).

Seedling mortality is high and sexual reproduction is sporadic. The likelihood of a particular plant flowering is reportedly increased by a preceding wet autumn and warm, wet winter free of sharp frosts (Pfeifer, 2004; see also Carey, 1996, 1998, 1999; Carey & Farrell, 2002; Carey *et al*., 2002; Pfeifer *et al*., 2006a, b, 2010), whereas spring droughts and/or frosts can result in the abortion of inflorescences. Putative climatic influences are discussed at greater length under 'Evidence for climatic drivers'.

Genetic diversity and putative migration routes

Himantoglossum hircinum supposedly colonised western Europe only after the last glaciation (e.g. Delforge, 2006) – in other words, within the last 11,500 years. The separation of the eastern *H. jankae-caprinum* group (*sensu* Molnár *et al*., 2012a) from the western *H. hircinum-adriaticum* group is tentatively estimated via the molecular clock rationale to have occurred much longer ago, at about 600 ka BP (Sramkó *et al*., 2013) – that is, in the middle of the Quaternary period. This date carries huge error bars and so should be treated with great caution. However, if interpreted literally, it would place the time of species divergence within the Cromerian interglacial period, which preceded three further glacial–interglacial cycles. Presumably, the separation of *H. adriaticum* from *H. hircinum* occurred appreciably more recently. Such observations suggest that, at least in theory, *H. hircinum* presents a good opportunity to use population genetic data to

study phylogeography – specifically, migration routes that reflect contractions to, and/or expansions from, glacial refugia.

A pioneering AFLP study of three isolated populations no more than 10 km apart in east-central Germany, marking the northeastern limit of the species' range (Heinrich & Voelckel, 1999; Heinrich, 2003), revealed greater variation within populations than between them (Pfeifer, 2004; Pfeifer & Jetschke, 2006). The results suggest that, at least in this region, *H. hircinum* is freely interbreeding at a local scale. When analysed at the level of individual plants, their data reliably distinguished one population from the other two, but the most localised of the two remaining populations was nested within the third, indicating a source–founder relationship between them.

Further developing this AFLP study, Pfeifer *et al*. (2009) later used both AFLP markers and plastid microsatellites to compare 20 populations (*ca* 200 plants) of *H. hircinum* distributed across much of western Europe. Little pattern was evident in the AFLP data, beyond greater divergence among western populations relative to those located further east. However, although plastid sequencing yielded only five haplotypes, they nonetheless tentatively suggested intriguing phylogeographic patterns. Having identified (albeit with limited justification) southern France as the species' centre of post-glacial migration, the authors argued that the "fitness" of populations (as crudely measured primarily by the approximate proportion of individuals in flower in the summer of 2007) decreased outwards in all directions from this region towards the margins of its geographic distribution. They also inferred the existence of two additional glacial refugia, in southern Italy and southern Spain, and three migratory pathways: westward into Iberia, northwestward into England, and northeastward into southern Germany.

Pfeifer *et al.* (2009) also suggested that English populations of *H. hircinum* may reflect northward migration from Iberia, as they share a predominance of their plastid haplotype 1. However, this assertion was based on analysis of only two English populations: "Burnham" (= our Berrow) and Sandwich. Our ITS ribotype data (Fig. 6) from 13 English populations better support Pfeifer *et al*.'s alternative hypothesis that the English lineage(s) migrated northwestward, possibly even originating from Pfeifer *et al*.'s "core" area in southeast France. The presence of category RGa3 ribotypes in southern Italy, Calais and 55 km across the English Channel in Hythe, Kent (Fig. 6a) is intriguing, as is the greater diversity of ribotypes within England along the Kent coast – a strip of land especially well-placed to receive seed from the frequent populations of *H. hircinum* distributed along the North French coast. Statistically, the diversity of ribotypes in general and "endemic" ribotypes in particular is equal in England and Continental Europe, arguing against very recent colonisation of England (and mirroring similar patterns of diversity documented in the biogeographically coincident orchid *Ophrys fuciflora* by Devey *et al*., 2009).

Lastly, we found no evidence to support the suggestion of Pfeifer *et al*. (2009, p. 2362) that *H. hircinum* occupied a fourth glacial refugium in the Balkans, but neither did we find evidence in support of our *a priori* theory that the Atlas Mountains of North Africa could have operated as a refugium; migration to the Atlas Mountains from Iberia appears more likely.

Evidence for climatic drivers of northward migration

The recent decrease in the official conservation status of the Lizard Orchid in the UK from Vulnerable to Near-Threatened seemingly flies in the face of its listing by Kull & Hutchings (2006) as one of the four orchid species to have declined most rapidly in the British Isles between the national botanical survey conducted in 1930–1969 and the subsequent survey conducted in 1987–1999 (yielding an estimated 83% decline in hectad

occurrences). However, much of the range expansion that was observed in the 1920s and 1930s involved sporadic and transient occurrences of single plants. Moreover, this expansionist phenomenon has recurred since the late 1990s (Fig. 1), the most recent errant plant emerging in 2009 on a remarkably unassuming urban roadside verge in west London convenient for the H28 bus (Fig. 3a). When happy with its immediate environment, *H. hircinum* shows invasive tendencies throughout its range, sometimes vigorously occupying roadside verges and garden lawns in the UK (Fig. 3b).

In England, the expansionist pulse that occurred during the 1920s increased population numbers from five to 30+ (Good, 1936; Carey, 1999), and that in the 1990s doubled population numbers from a previous medium-term average of about ten (Carey, 1999; Farrell & Carey, 1999). Although Foley & Clarke (2005) argued that about 90% of occurrences in the UK have been single flowering plants, the historical data accumulated by Carey (1999) suggest that this figure is exaggerated. The now sizeable Sandwich population is inferred to have spawned several satellite populations within 15 km radius during the late 1990s (Carey *et al*., 2002). Such 'embryonic' populations are obviously very vulnerable to rapid extirpation, not least through botanical collecting; Carey (1999) estimated that 20% of UK populations had been lost to collectors.

Himantoglossum hircinum has benefited from two detailed programmes designed to appraise in detail its responses to perceived climate change, the first spearheaded by Peter Carey and the second by Marion Pfeifer. To summarise briefly a vast panoply of data, the probability of flowering is reportedly increased by a preceding wet autumn and warm, wet, frost-free winter – in other words, by the imposition of a broadly Mediterranean climate (Carey, 1996, 1998, 1999; Carey & Farrell, 2002; Carey *et al*., 2002; Pfeifer, 2004; Pfeifer *et al*., 2006a, b, 2010), whereas spring droughts and/or frosts are detrimental to flowering – conclusions largely congruent with those drawn from largely anecdotal evidence much earlier by Good (1936). It is worth noting that conditions optimal for the progressive growth of seedlings to reach flowering size (a particular challenge to *H. hircinum* according to the above authors) may not equate with those that are optimal for prompting flowering *per se*. For example, one author argued that unusually high summer rainfall aids seedlings but represents a threat to more mature tubers. Locally, the interaction of microclimate with soil type appears to be of greatest consequence to the health of populations.

In southern German populations, the combination of plant size and weather conditions has been estimated to explain 82% of the observed major fluctuations in numbers of flowering plants within particular populations (Pfeifer, 2004). The proportion of flowering plants in one substantial population averaged 5% and rarely exceeded 10% in any one year. Moreover, *ca* 70% of individuals flowered only once during the 25-year survey, suggesting that producing such large inflorescences is resource intensive, and helping to explain the transient occurrences of single plants close to the range margin of the species. It may be pertinent that three of the ten plants measured by us at Newmarket possessed only the seven leaves shown by Pfeifer (2004) to be the minimum number required to provide a probability exceeding 30% of flowering being initiated during the forthcoming season.

Expanding the geographical coverage of their population sampling, Pfeifer *et al.* (2010) suggested that front-edge and rear-edge populations of a sweeping climatically-driven migration experience significantly different demographic influences and consequently require significantly different conservation strategies. These insights also have a broader significance, because they could permit the development of demographic models that have the potential to become predictive if they can be rendered sufficiently sophisticated (cf. Carey & Brown, 1994; Carey & Farrell, 2002; Pfeifer *et al*., 2006a).

Even if the distribution of *H. hircinum* and its relatives is indeed particularly responsive to climate change, their phenology appears to us to be more robust. In Hungary at least, *H. adriaticum* – the sister species of *H. hircinum* – has proven to be less susceptible than most species of Orchidinae to phenological shifts in presumed responses to climate change (Molnár *et al*., 2012b). In this context, it is interesting to note that the typical altitude of *H. hircinum* populations appears to increase with decreasing latitude. No population in the UK exceeds 200 m asl (Carey & Farrell, 2002), whereas the populations studied by Pfeifer *et al*. (2009) in France and Germany occurred at 200–800 m asl and those in the southern Italian and Spanish outliers at (700–)900–1150 m asl. Occurring as high as 1650 m asl, the Moroccan population studied by us approached the maximum altitude attained by *H. hircinum* (1800 m asl according to Delforge, 2006); moreover, two further small populations of *H. hircinum* examined by the authors in the Ifrane–Azrou region of Morocco similarly occurred at 1620 and 1680 m asl. Such altitudinal responsiveness could be taken as further (albeit circumstantial) evidence of the sensitivity of the species to its environmental conditions; even at a latitude as low as 33°N, at least some populations of *H. hircinum* do not reach peak flowering until early June.

Lastly, the suggestion of Carey (1999) and Pfeifer (2004) that the fact that individuals of *H. hircinum* occur in clumped aggregates with metapopulations is due to seeds typically falling close to the seed-parents could instead reflect a need for the seeds to share their parents mycorrhizal partners (cf. Carey & Farrell, 2002). Under this hypothesis, intensive filtration as a result of mycorrhizal specificity would prevent most attempts at medium- and long-distance dispersal by airborne seeds (e.g. Bateman, 2006). This hypothesis is seemingly rendered less likely by a comparatively early survey of mycorrhizae in *H. hircinum* that reported 15 strains of endomycorrhizae in the roots (Gäumann *et al*., 1961), thus suggesting that this species may be a generalist when forming subterranean symbioses. However, Carey & Farrell (2002) later questioned whether the isolated fungi played a genuinely mutualistic role with the orchid. Further study of the mycorrhizae of *H. hircinum* using more modern approaches is urgently required as an essential contribution to any interpretation of climatic influences on its distribution and migration.

***Possibility that cryptic species exist within* H. hircinum**

Returning to the accumulated molecular data, recent analyses suggest that there is an approximately equal probability that *H. hircinum* and *H. adriaticum* diverged in western or central Europe (Sramkó *et al*., 2013). The reliable genetic cohesion of *H. hircinum* evident from plastid and low-copy nuclear *LEAFY* sequences suggests that the inability to confidently distinguish *H. hircinum* from other species of *Himantoglossum s.s.* using ITS sequences is more likely to reflect incomplete lineage sorting than more recent hybridisation (Sramkó *et al*., 2013). Considered together, these phylogenetic patterns suggest that *H. hircinum* has existed for longer than at least some of the other species of *Himantoglossum s.s.* that occur further east. These results encourage the belief that contentious division through the last *ca* 35 years of the former *H. hircinum s.l.* into several species is largely justified, but they also raise an additional question; do multiple cryptic species (Bateman, 2011; Bernardo, 2011) still exist within *H. hircinum s.s.*?

The chromosome number that is by far the most frequently reported in *Himantoglossum s.l.* is $2n = 36$ (reviewed by Pridgeon *et al*., 1997; Neiland *et al*., 2001; Bateman *et al*., 2003) – the diploid number that typifies the multi-genus "$2n = 36$" clade of which *Himantoglossum s.l.* is the earliest divergent member (Pridgeon *et al*., 1997; Bateman *et al*., 2003). However, reports of chromosome numbers in *H. hircinum* initially

appeared conflicting; most authors stated that 2*n* = 36 (Bournérias & Prat, 2005; Delforge, 2006; Claessens & Kleynen, 2011), whereas others stated that 2*n* = 24 (Sell & Murrell, 1996; Foley & Clarke, 2005), perpetuating a count published by Heusser (1915). Yet others hedged their bets by offering 2*n* = 24, 36 or *n* = 12, 18 (Moore, 1980; Bianco *et al*., 1987; Neiland *et al*., 2001; Carey & Farrell, 2002; Stace, 2010). A more detailed examination of 12 Italian karyotypes of *H. adriaticum* by D'Emerico *et al.* (1993) suggested the additional presence of a B chromosome in one of the plants and revealed the majority of the chromosomes to be comparatively symmetrical, heterochromatin-poor metacentrics.

More recent chromosomal studies have provided greater detail and insight. Six assessments presently listed on the IPCN chromosome database give values of 2*n* = 36 (cf. D'Emerico *et al*., 1990) but include an additional assessment from Salamanca, Spain of 2*n* = 18 (cf. Bernardos & Amich, 2002). Interestingly, genome size (rather than chromosome number) determinations of two plants of *H. hircinum* yielded values of C = 6.1 and 12.2 pg (Leitch *et al*., 2009), thus similarly suggesting the existence within *H. hircinum* s.s of two ploidy levels. In contrast, no further evidence has come to light of plants with the less credible complement of 2*n* = 24.

Particular uncertainty hangs over the geographical outlier in southern Italy and Sicily of populations that today are widely attributed to *H. hircinum* despite being separated from its distributional centre in France by a swathe of populations attributed to its sister-species, *H. adriaticum*. Firstly, our ITS ribotype data show the presence of a unique and comparatively divergent ribotype group in Sicily (Ga4: Fig. 6), whereas ribotypes found in adjacent populations of *H. adriaticum* correspond with the Ga1 ribotypes that characterise the majority of populations of *H. hircinum* elsewhere in Europe. Moreover, the sole southern Italian locality studied by Pfeifer *et al*. (2009), approximating our Calvello locality, was the only population to be dominated by their plastid haplotype 4. This insight reinforces the unique ITS ribotypes observed by us in the same geographical outlier, rather than supporting the suggestion of Pfeifer *et al.* (2009) that haplotype 4 may have been acquired through hybridisation with *H. adriaticum* populations now contiguous with the northern margin of this outlier. Thus, at present, we regard as questionable the attribution of southern Italian populations to *H. hircinum s.s.*

Denser sampling of populations of both putative species in southern Italy is clearly warranted, combined with acquisition of morphometric data from the region (sadly, none are presently available). It seems likely that microsatellites currently being developed by Sramkó and colleagues will help to solve this and other riddles still posed by *Himantoglossum s.l.*

Conclusions

Data from studies of population demographics (e.g. Carey, 1999; Pfeifer *et al*., 2006a) and pollination (summarised by Claessens & Kleynen, 2011) are in accord with our genetic data in suggesting that the Lizard Orchid is typical of tuberous-rooted, non-pseudocopulatory species of European Orchideae. In short, the ontogenetic pattern and mature size of the hyper-elongated central labellar lobe and crenulated lateral lobes of *Himantoglossum s.s*. appear in retrospect to be its most remarkable feature, though detailed surveys of ploidy levels via flow cytometry (cf. the study of *Gymnadenia* by Trávníček *et al*., 2012) and of associated mycorrhizae (cf. the study of *Dactylorhiza* by Jacquemyn *et al*., 2012) might yet prove instructive. Irrespective of particular theories of plant migration in response to climate change, research conducted on *H. hircinum* during the last two decades has clearly demonstrated the exceptional value of conducting

relatively long-term monitoring experiments (cf. Pfeifer, 2004; Bateman, 2011, 2013). Nonetheless, achieving less ambiguous interpretations will evidently require in-depth investigations of many more plant species, adding to the pioneering work already conducted on *H. hircinum*.

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[CAPTIONS TO FIGURES]

Figure 1 Distribution of *Himantoglossum hircinum* in the British Isles, showing the localities sampled for DNA (red spots) and for morphometric study (letters: S = Sandwich, N = Newmarket). Base map reproduced from Preston *et al*. (2002, p. 854).

Figure 2 Representative plants of *Himantoglossum hircinum* from the Sandwich (c, g), Newmarket (f) and Ifrane (b, e) populations, plus the transient Headley plant (a, d). (e)–(g) are reproduced at the same scale (vertical dimension = 65 mm). Images: (f) = D.M.T. Ettlinger, (g) = B.G. Tattersall, remainder = R.M. Bateman.

Figure 3 Habitats of *Himantoglossum hircinum* at Sandwich (d), Newmarket (e) and Ifrane (b), together with (c) plants invading a garden lawn at Sandwich and (a) a transient 'rogue' plant (centre foreground) that appeared on an urban roadside verge in Isleworth in 2009–2011. Images: (e) = I. Denholm, remainder = R.M. Bateman.

Figure 4 Silhouettes of mounted flowers of plants measured for the present morphometric survey from the three study populations of *Himantoglossum hircinum*. (a) = Sandwich, (b) = Newmarket, (c) = Ifrane. Scale bar = 20 mm.

Figure 5 Parsimony tree of ITS sequences for 40 accessions of *Himantoglossum s.l.* (numbers per taxon are given in parentheses) plus three accessions of *Steveniella* as outgroups. All internal branches received strong statistical support (bootstrap support = 99–100%, posterior probability = 1.0) except that arrowed (BS = 75%, PP = <0.5).

Figure 6 Distribution across western Europe of ITS ribotypes observed in *Himantoglossum hircinum* through direct sequencing; eight groups are delimited by single-nucleotide polymorphisms and three through length-variable polymorphisms (L), together with a putative pseudogene (PG) (see also Sramkó et al., 2013). Each spot represents a single plant; overlapping spots indicate multiple accessions derived from the same locality. Scale bar is 18 km for Kent and East Sussex (a), 100 km for England (b) and 500 km for western Europe (c). Base maps courtesy of Google Earth.

Figure 7 Plot of the first two principal coordinates for 23 individuals of *Himantoglossum hircinum* from three study populations.

Figure 8 Plot of stem diameter versus stem height for 23 individuals of *Himantoglossum hircinum* from three study populations. Regression lines and r^2 values are also shown.

Figure 9 Plot of 'arm' length versus 'leg' length for 23 individuals of *Himantoglossum hircinum* from three study populations.

Figure 10 Artificially coloured, composite scanning electron micrographs depicting a series of three developmental stages (a–c) of buds excised from a plant of *Himantoglossum hircinum* cultivated by Richard Manuel. The two lateral petals and

all three sepals have been removed. Mauve = ovary, grey = base of gynostemium, pale yellow = bursicles and connective, dark yellow = auricles, blue = labellar spur, green = lateral labellar lobes, red = central labellar lobe. Scale bar = 500 µm. Images: P.J. Rudall.

Figure 11 Scanning electron micrographs of (a) an entire immature labellum and (b) the strongly papillate central region of a mature labellum of a bud and flower respectively excised from a plant of *Himantoglossum hircinum* cultivated by Richard Manuel. Scale bars = 100 µm. Images: P.J. Rudall.

Colour figure

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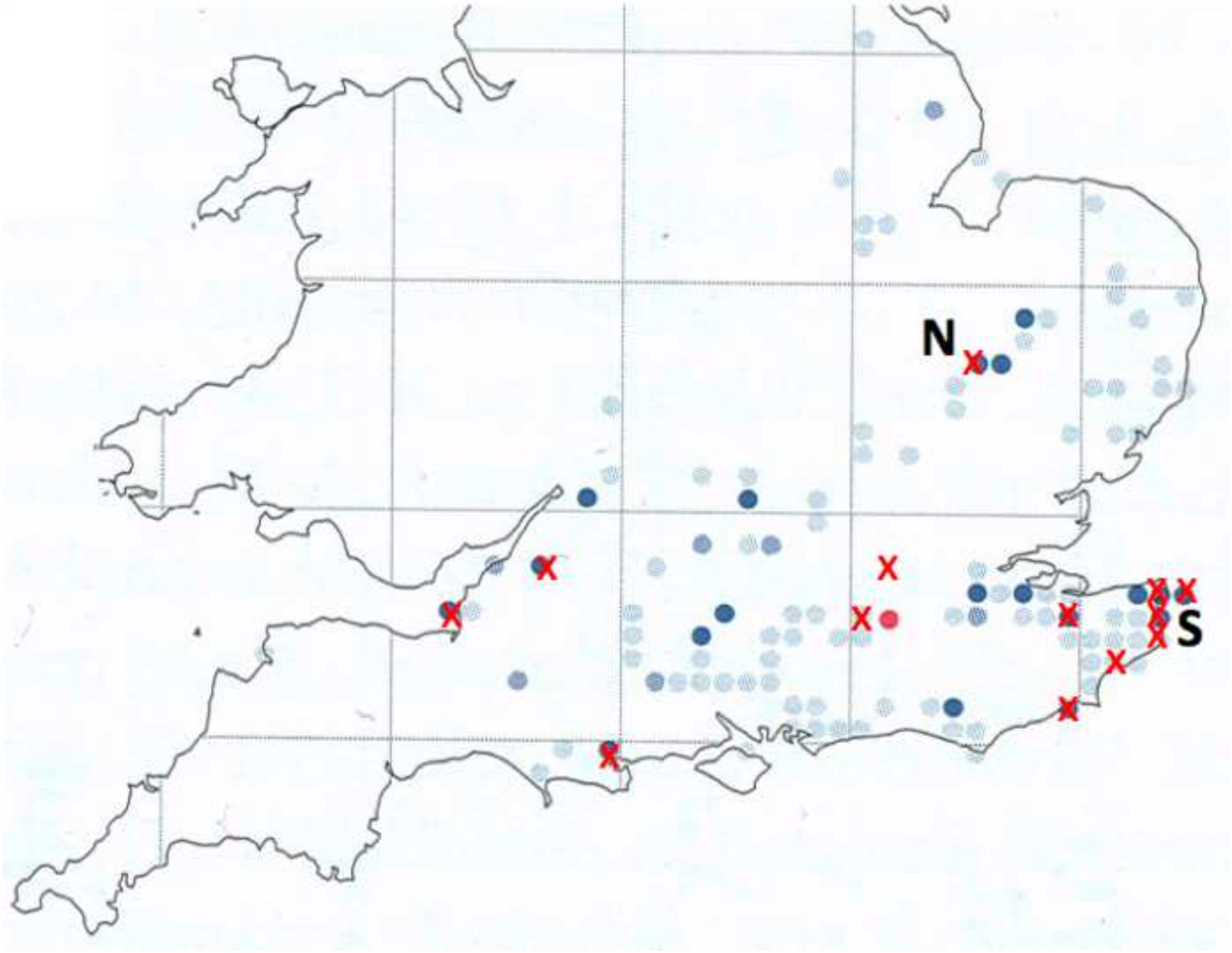

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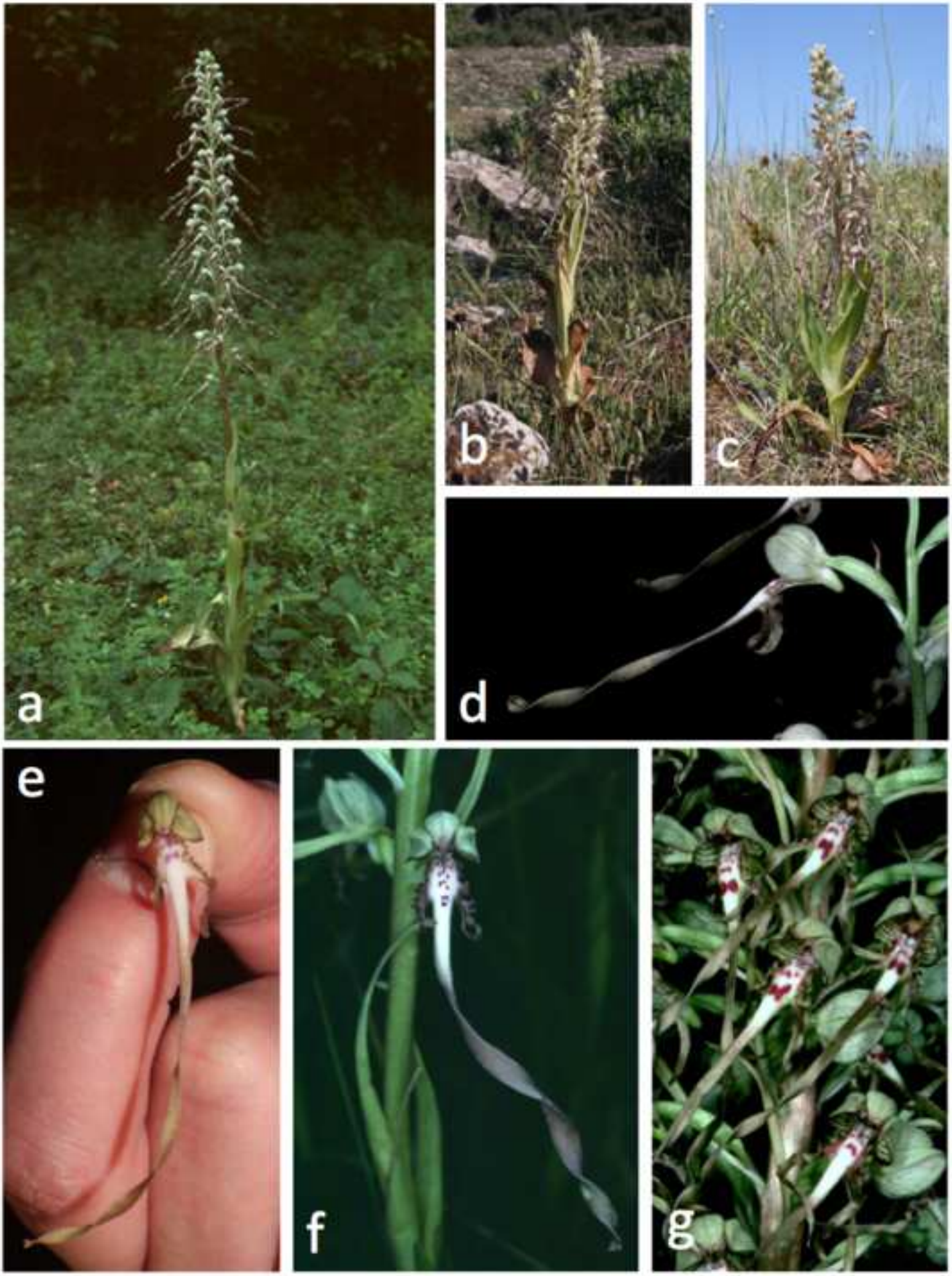

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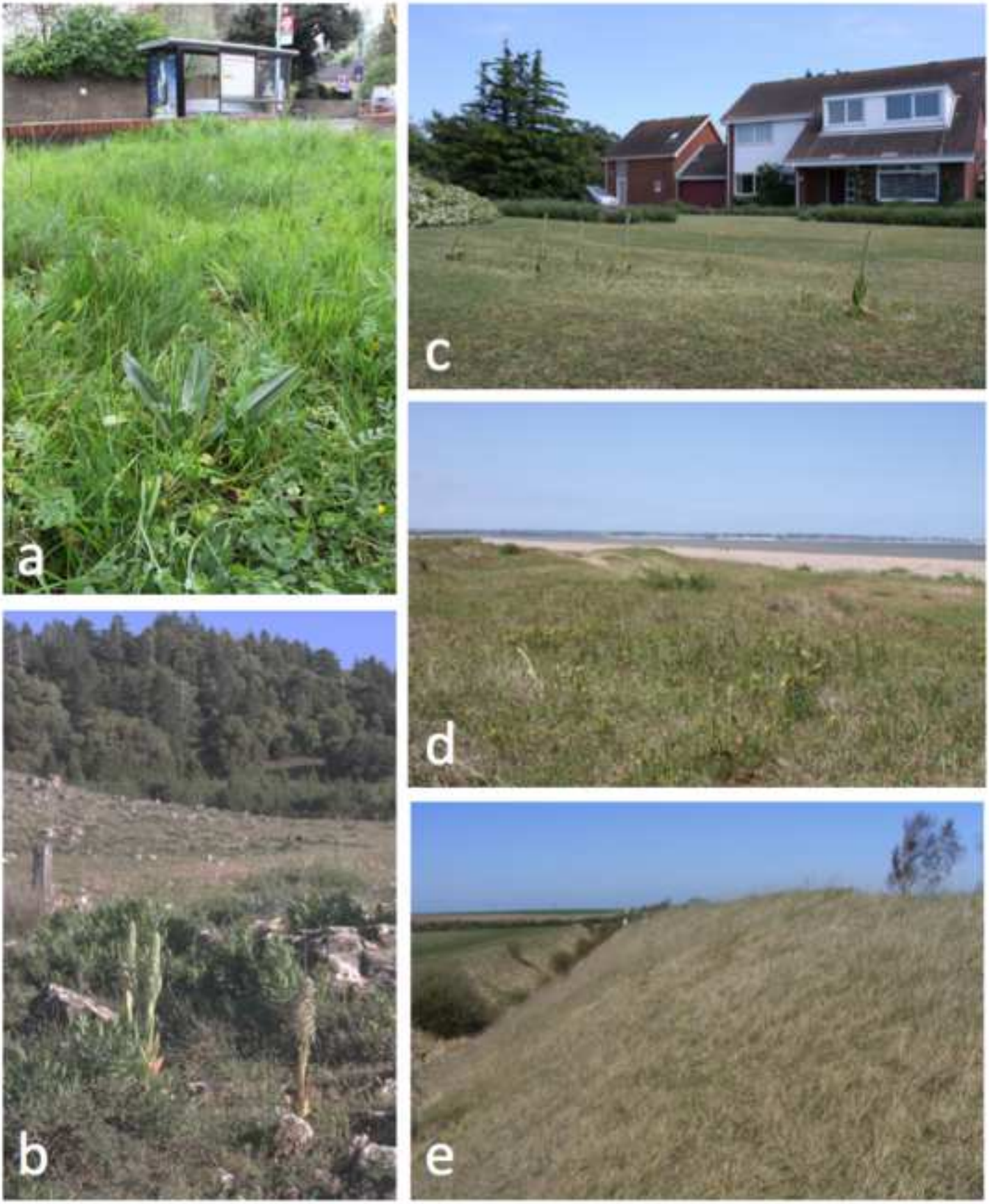

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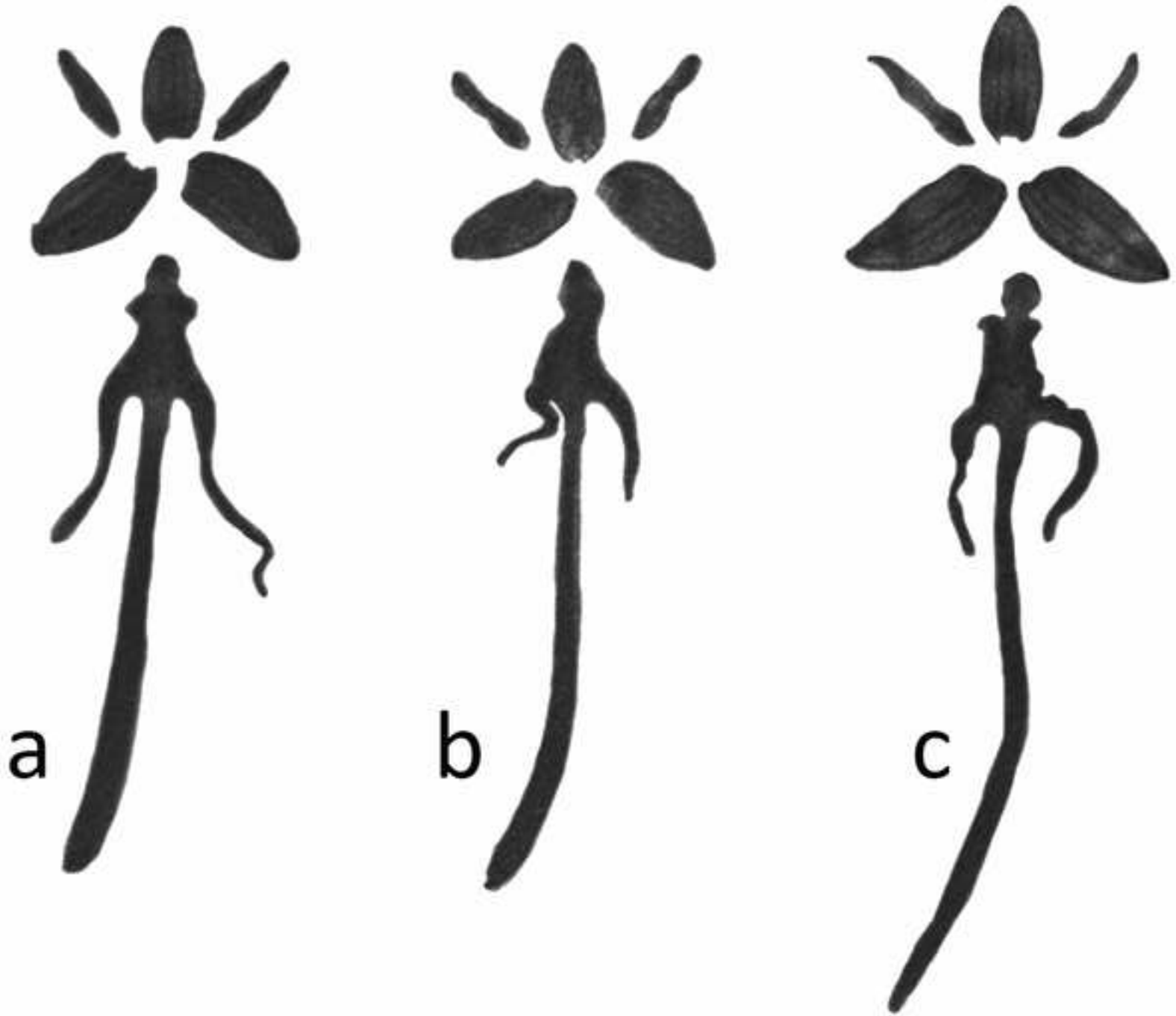

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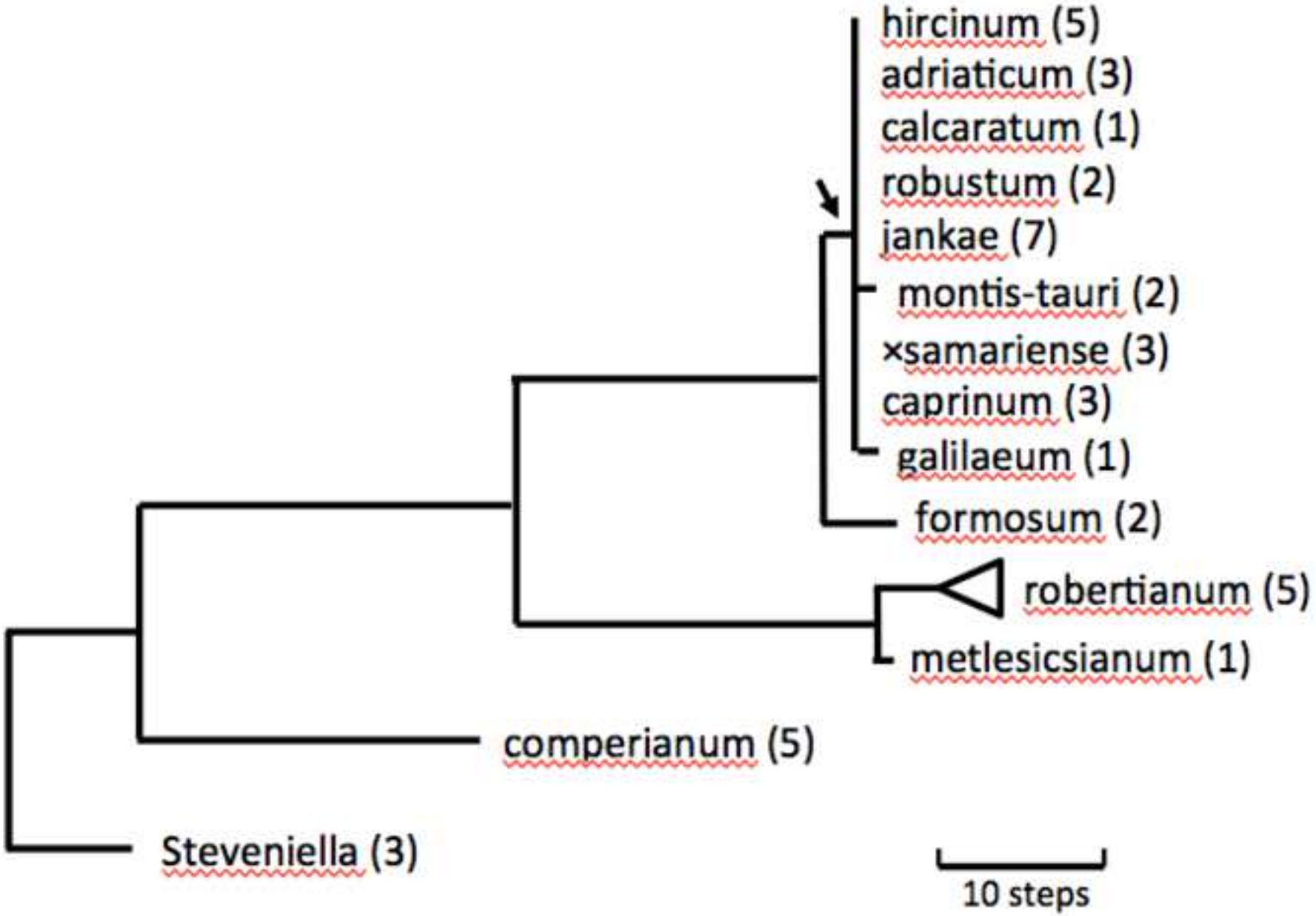

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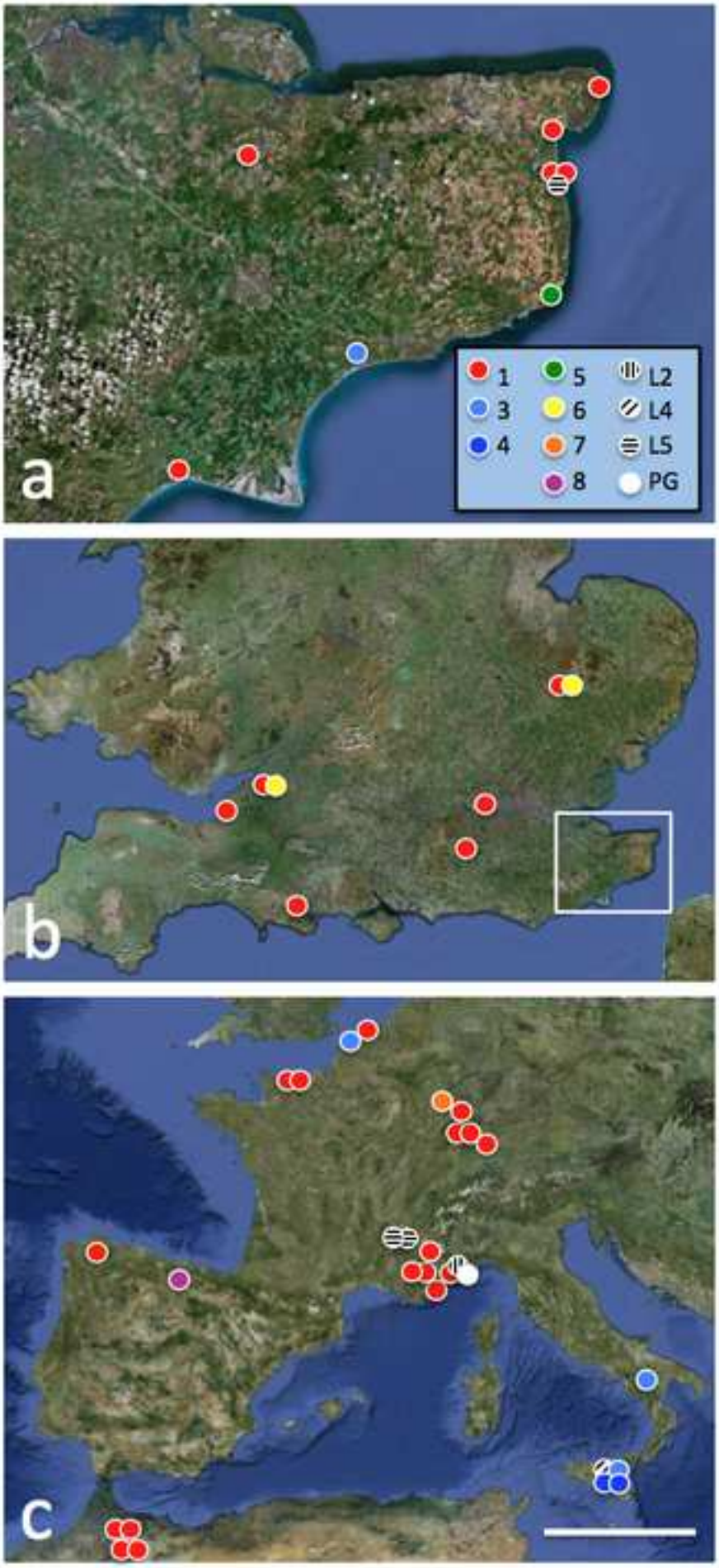

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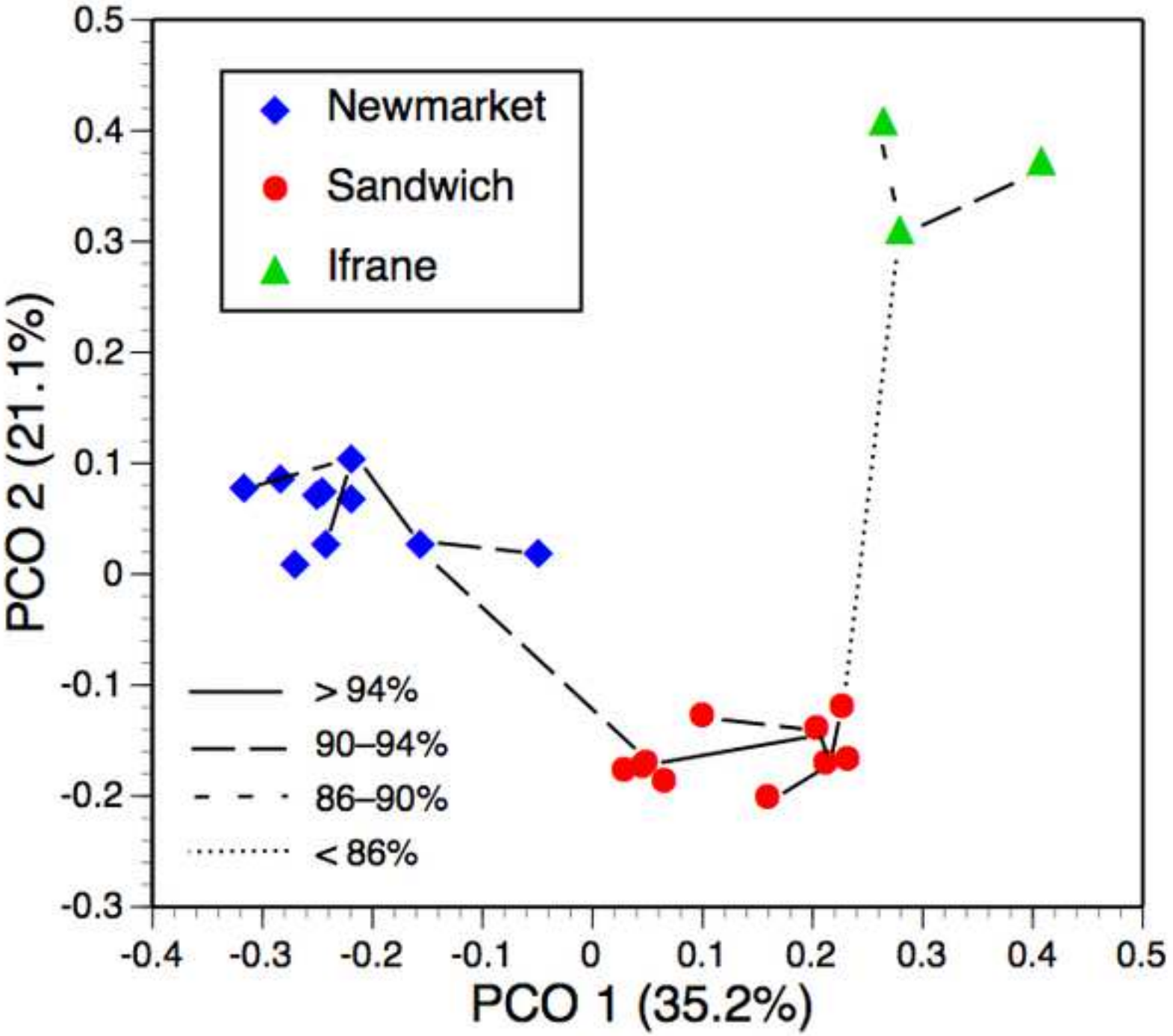

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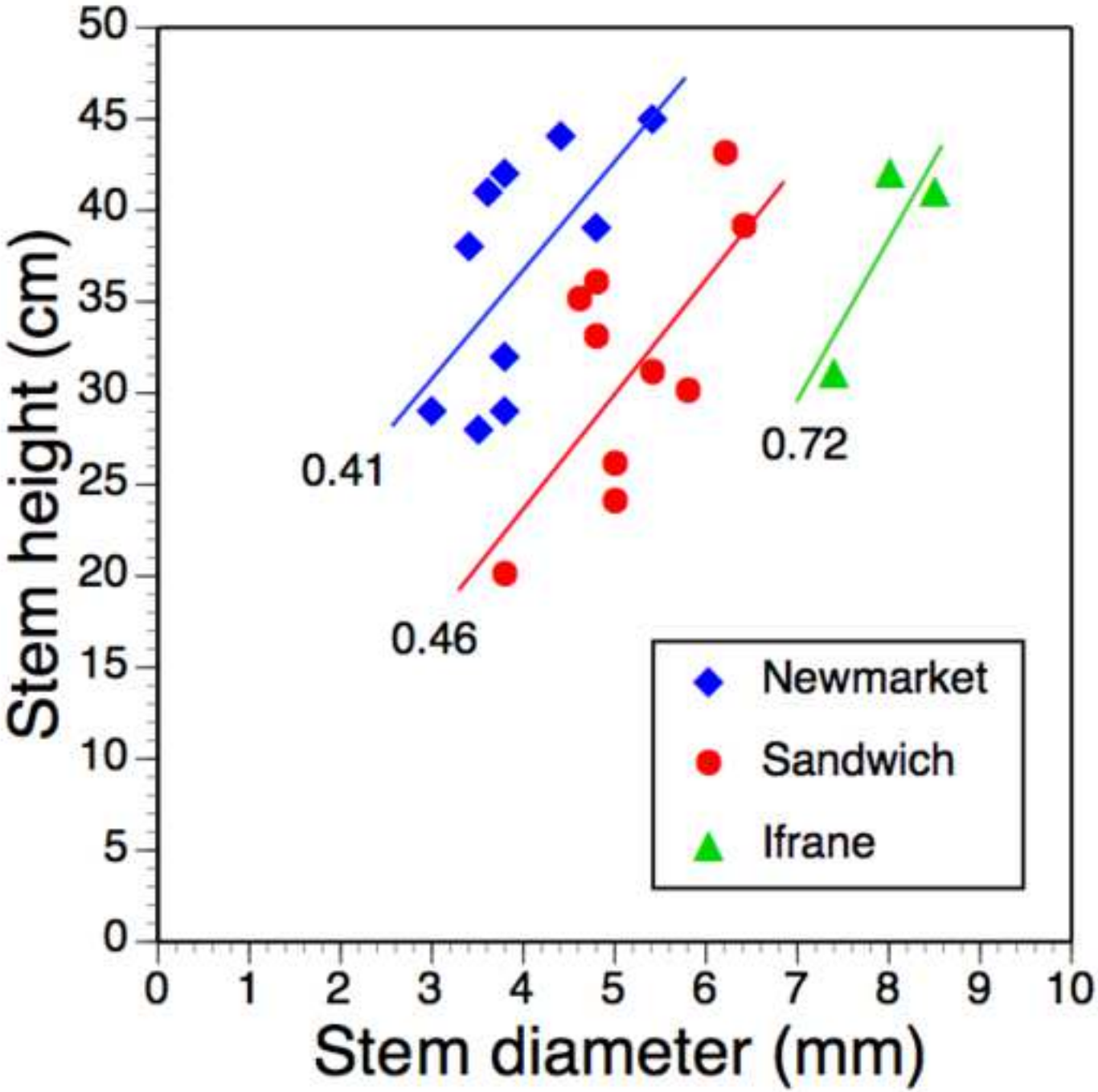

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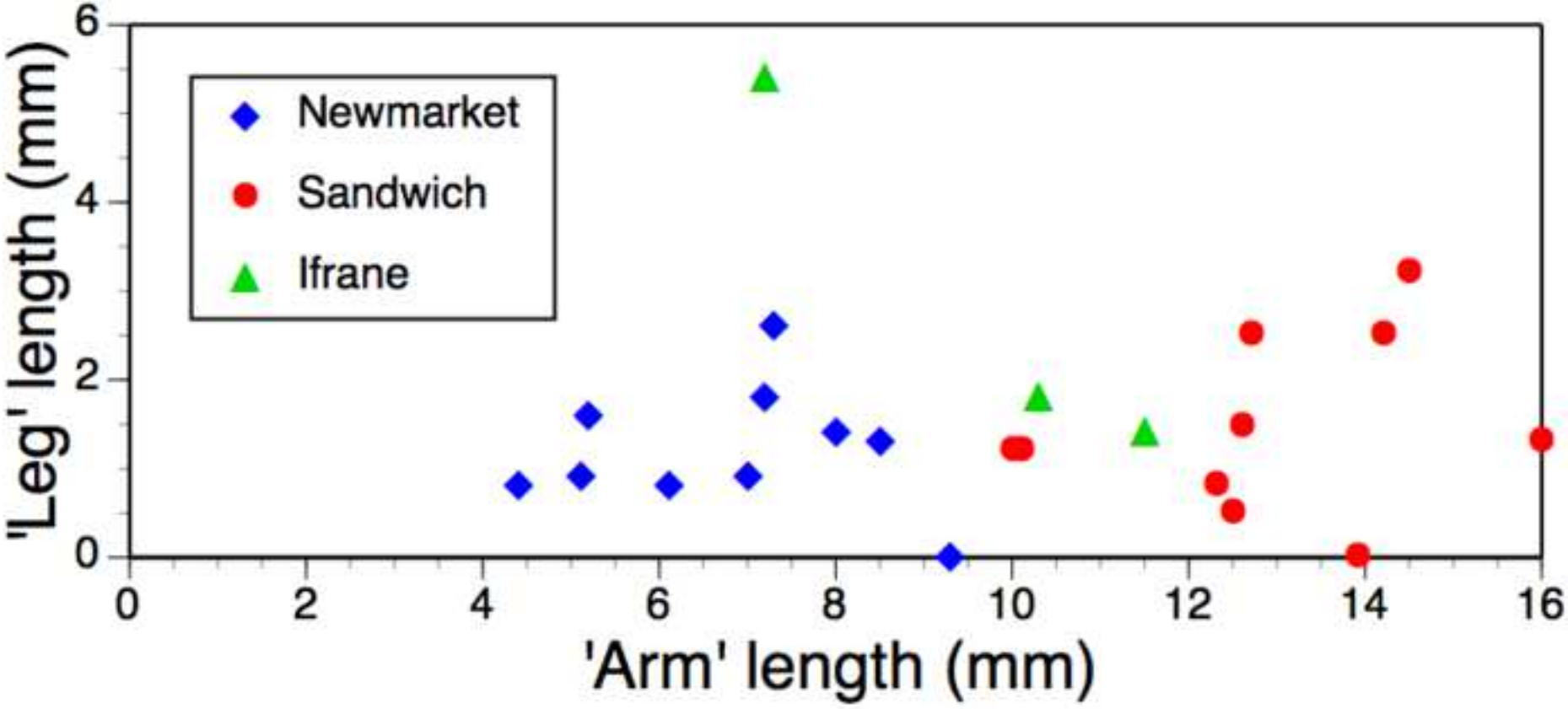

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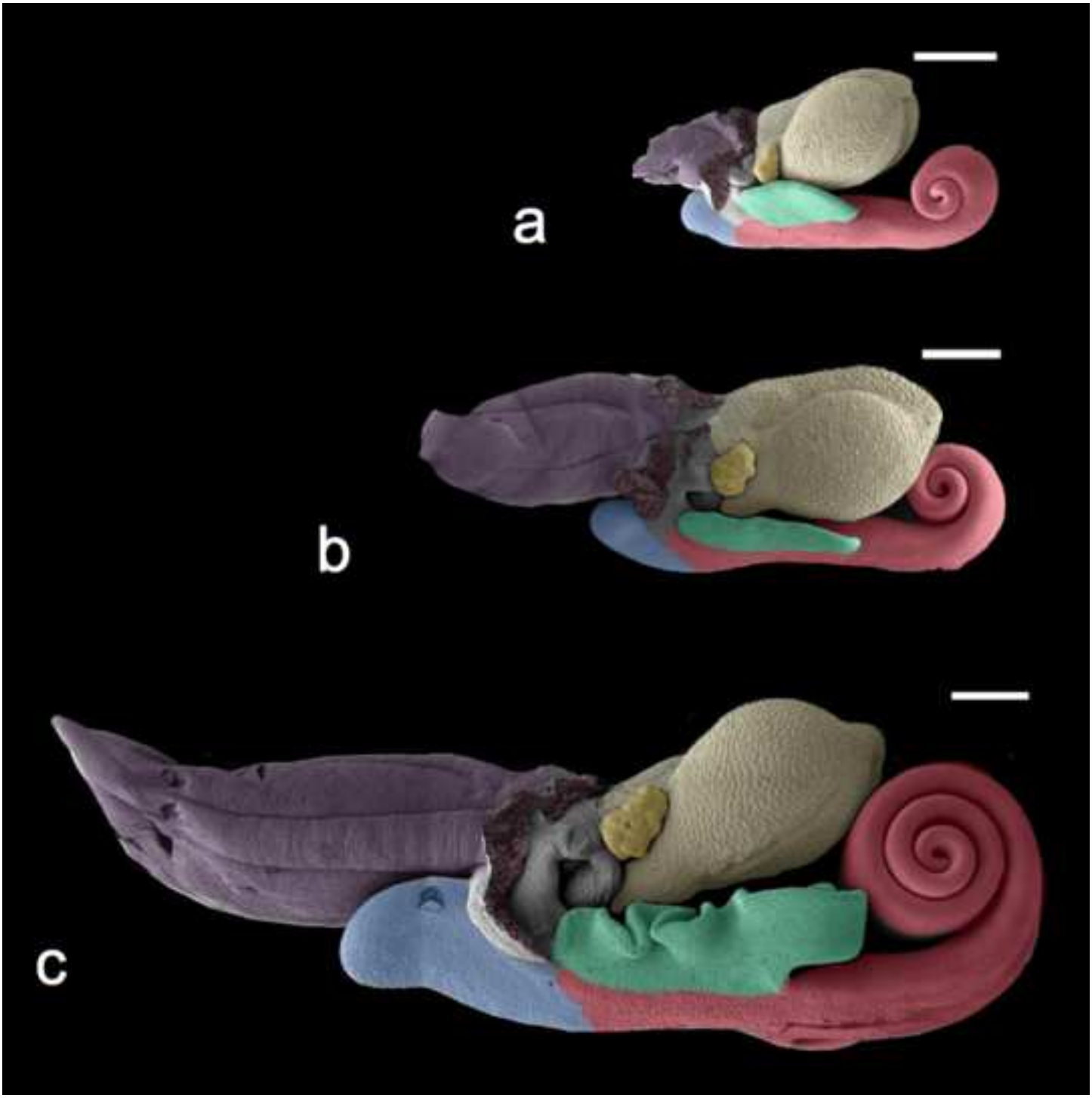

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